Microbulbifer celer sp. nov., isolated from a marine solar saltern of the Yellow Sea in Korea

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A Gram-negative, non-motile, rod-shaped, *Microbulbifer*-like bacterial strain, ISL-39^T, was isolated from a marine solar saltern of the Yellow Sea in Korea and was subjected to a polyphasic taxonomic investigation. Strain ISL-39^T grew optimally at pH 7.0–8.0 and 37 °C. It contained Q-8 as the predominant ubiquinone and iso-C_{15:0}, C_{16:0} and iso-C_{17:0} as the major fatty acids. The DNA G + C content was 57.7 mol%. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain ISL-39^T belonged to the genus *Microbulbifer*. Strain ISL-39^T exhibited 16S rRNA gene sequence similarity values of 94.7–97.5 % with respect to the type strains of four recognized *Microbulbifer* species. DNA–DNA relatedness data and the differential phenotypic properties and phylogenetic distinctiveness of ISL-39^T make this strain distinguishable from the recognized *Microbulbifer* species. On the basis of the phenotypic, phylogenetic and genetic data, strain ISL-39^T represents a novel species of the genus *Microbulbifer*, for which the name *Microbulbifer celer* sp. nov. is proposed. The type strain is ISL-39^T (=KCTC 12973^T=CCUG 54356^T).

The genus *Microbulbifer* was erected by González *et al.* (1997) with the description of a single species, *Microbulbifer hydrolyticus*. Subsequently, two further *Microbulbifer* species, *Microbulbifer salipaludis* (Yoon *et al.*, 2003a) and *Microbulbifer maritimus* (Yoon *et al.*, 2004), were described. *Pseudomonas elongata* was reclassified into the genus *Microbulbifer*, as *Microbulbifer elongatus* (Yoon *et al.*, 2003b). In this study, we report on the taxonomic characterization of a *Microbulbifer*-like bacterial strain (ISL-39^T) that was isolated from a marine solar saltern of the Yellow Sea in Korea.

Strain ISL-39^T was isolated by means of the standard dilution plating technique, at 25 °C on marine agar 2216 (MA; Difco) supplemented with 6 % (w/v) NaCl. The type strains of four *Microbulbifer* species were used as reference strains for DNA–DNA hybridization: *M. hydrolyticus* DSM 11525^T and *M. elongatus* DSM 6810^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and *M. salipaludis* SM-1^T and *M. maritimus* TF-17^T were obtained from previous studies (Yoon *et al.*, 2003a, 2004). The morphological, physiological and biochemical characteristics of strain ISL-39^T were investigated using routine cultivation on MA at 37 °C. The cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy. Flagellation was investigated by using a CM-20 transmission electron microscope (Philips) with

cells from exponentially growing cultures: for this purpose, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and on MA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Growth in the absence of NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded. Growth at various NaCl concentrations was investigated in marine broth 2216 (Difco) or trypticase soy broth (Difco). Growth at various temperatures (4–50 °C) was measured on MA. Catalase and oxidase activities and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and nitrate reduction were investigated as described previously (Lanyi, 1987), except that artificial seawater was used for the preparation of media. The artificial seawater contained the following (l⁻¹ distilled water): 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). H₂S production was tested as described previously (Bruns *et al.*, 2001). Susceptibility to various antibiotics was investigated on MA plates by using antibiotic discs with the following concentrations: 100 U polymyxin B, 50 µg streptomycin, 20 U penicillin G, 100 µg chloramphenicol, 10 µg ampicillin, 30 µg cephalothin, 30 µg gentamicin, 5 µg novobiocin,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ISL-39^T is EF486352.

30 µg tetracycline, 30 µg kanamycin, 15 µg lincomycin, 15 µg oleandomycin, 30 µg neomycin and 100 µg carbenicillin. Acid production from carbohydrates was tested as described by Leifson (1963). The utilization of various substrates for growth was determined as described by Yurkov *et al.* (1994). Enzyme activities were determined by using the API ZYM system (bioMérieux).

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from cultivation in marine broth 2216 at 37 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), except that RNase T1 was used in combination with RNase A to minimize contamination from RNA. The 16S rRNA gene was amplified by using PCR with two universal primers, as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and the phylogenetic analysis were performed as described by Yoon *et al.* (2003a). Isoprenoid quinones were investigated as described by Komagata & Suzuki (1987), using reversed-phase HPLC. For cellular fatty acid analysis, cell mass was harvested from MA plates after cultivation of strain ISL-39^T for 2 days at 37 °C. The fatty acids were extracted and the fatty acid methyl esters prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by using the method of Tamaoka & Komagata (1984), with the modification that the DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically according to the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values were quoted as the DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain ISL-39^T are given in the species description (see below) or in Table 1. The almost-complete 16S rRNA gene sequence of strain ISL-39^T determined in this study comprised 1491 nt, representing approximately 96% of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analysis revealed that strain ISL-39^T was most closely related phylogenetically to members of the genus *Microbulbifer*. In the phylogenetic tree based on the neighbour-joining algorithm, strain ISL-39^T fell within the clade comprising the *Microbulbifer* species (Fig. 1). Strain ISL-39^T exhibited 16S rRNA gene sequence similarity values of 97.5, 97.5, 96.2, 94.7 and 94.5% with respect to the type strains of *M. hydrolyticus*, *M. salipaludis*, *M. elongatus*, *M. maritimus* and '*Microbulbifer arenaceus*', respectively. The values for sequence similarity with respect to other species used in the phylogenetic analysis were below 90.8%. The chemotaxonomic properties of ISL-39^T confirm the phylogenetic results, indicating an affiliation with the genus

Microbulbifer (González *et al.*, 1997; Yoon *et al.*, 2003a, b, 2004). The predominant isoprenoid quinone detected in strain ISL-39^T was Q-8 (at a peak area ratio of approximately 92%). The fatty acid profile of strain ISL-39^T showed the presence of large amounts of branched, straight-chain, unsaturated, hydroxyl and cyclo fatty acids; the major components (>10% of total fatty acids) were iso-C_{15:0}, C_{16:0} and iso-C_{17:0} (Table 2). This fatty acid profile was similar to those of the *Microbulbifer* species, although there were differences in the proportions of some fatty acids, probably because of differences in cultivation conditions and extraction procedures (Table 2). The DNA G+C content of strain ISL-39^T was 57.7 mol%. Strain ISL-39^T exhibited DNA–DNA relatedness values of 7–16% with respect to the type strains of four recognized *Microbulbifer* species, indicating that ISL-39^T represents a different genomic species (Wayne *et al.*, 1987). Strain ISL-39^T is distinguishable from the recognized *Microbulbifer* species through differences in several phenotypic characteristics, as shown in Table 1. The phylogenetic and genetic distinctiveness of ISL-39^T, together with the differential phenotypic properties, are sufficient to permit the allocation of this strain to a species that is separate from the recognized *Microbulbifer* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strain ISL-39^T represents a novel species of the genus *Microbulbifer*, for which the name *Microbulbifer celer* sp. nov. is proposed.

Description of *Microbulbifer celer* sp. nov.

Microbulbifer celer (ce'ler. L. masc. adj. *celer* rapid, pertaining to fast growth).

Cells are Gram-negative and rod-shaped (0.2–0.4 × 0.8–3.5 µm). Colonies on MA are circular to slightly irregular, slightly convex, smooth, glistening, greyish yellow in colour and 3.0–4.0 mm in diameter after 2 days incubation at 37 °C. Growth occurs at 10 and 48 °C, but not at 4 or 49 °C. The optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 5.0, but not at pH 4.5. Growth occurs in the presence of 15% (w/v) NaCl, but not in the absence of NaCl or in the presence of more than 16% (w/v) NaCl. Anaerobic growth does not occur on MA or on MA supplemented with nitrate. Hypoxanthine and Tweens 20, 40 and 60 are hydrolysed, but urea, L-tyrosine and xanthine are not. Acetate and pyruvate are utilized, but D-glucose, D-fructose, D-galactose, D-cellobiose, D-mannose, trehalose, D-xylose, L-arabinose, sucrose, maltose, citrate, succinate, benzoate, L-malate, salicin, formate and L-glutamate are not utilized. Acid is produced from melibiose, but not from D-mannitol, D-melezitose, D-raffinose, L-rhamnose, D-ribose, D-sorbitol or *myo*-inositol. Susceptible to chloramphenicol, kanamycin, neomycin and novobiocin and weakly susceptible to oleandomycin and polymyxin B, but not to ampicillin, carbenicillin, cephalothin, gentamicin, lincomycin, penicillin G, streptomycin or tetracycline. The predominant ubiquinone is Q-8. The major fatty acids (>10% of total fatty acids) are iso-C_{15:0}, C_{16:0} and

Table 1. Differential phenotypic characteristics of strain ISL-39^T and *Microbulbifer* species

Strain Species: 1, ISL-39^T, data from this study; 2, *M. hydrolyticus*, data from González *et al.* (1997), Yoon *et al.* (2003a) and this study; 3, *M. salipaludis*, data from Yoon *et al.* (2003a) and this study; 4, *M. elongatus*, data from Humm (1946) and this study; 5, *M. maritimus*, data from Yoon *et al.* (2004). All of the species are positive for the following: catalase and oxidase activities (data for *M. hydrolyticus* and *M. elongatus* from this study), alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase; hydrolysis of starch and Tween 80 (data for *M. hydrolyticus* and *M. elongatus* from this study); growth at pH 6.0 and 10 % NaCl (data for *M. hydrolyticus* and *M. elongatus* from this study); acid production from D-cellobiose. All species are negative for spore formation, Gram-staining, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase, α -fucosidase, acid production from D-fructose and D-mannose and growth at 0 % NaCl. +, Positive reaction; –, negative reaction; w, weakly positive reaction.

Characteristic	1	2	3	4	5
Cell morphology	Rods	Rods	Rods	Cocci or rods	Rods
Colony colour	Greyish yellow	Cream	Greyish yellow	Yellowish brown	Yellowish brown
Motility	–	–	–	+	–
Reduction of nitrate to nitrite	–	–*	+†	–*	–
Hydrolysis of:					
Aesculin	+	+*	–	+*	–
Agar	–	–*	w	+*	–
Casein	+	+*	w	+*	+
Gelatin	–	+*	–†	+*	+
Acid production from:†					
L-Arabinose	+	+	–	+	–
D-Galactose	+	–	–	+	–
D-Glucose	–	+	+	+	–
Lactose	+	–	–	–	–
Maltose	–	+	+	+	+
Sucrose	–	–	–	w	–
Trehalose	+	–	–	–	+
D-Xylose	+	+	–	+	–
N-Acetyl- β -glucosaminidase (API ZYM)‡	+	–	+	+	–
Optimum growth temperature (°C)	37	37	37	25–30	37
DNA G + C content (mol%)	57.7	58.2*	59	58.2*	59.9

*Data for *M. hydrolyticus* and *M. elongatus* from this study.

†Data for *M. elongatus* from this study.

‡Data for *M. salipaludis* from this study.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain ISL-39^T, *Microbulbifer* species and some other related taxa. Bootstrap percentages (based on 1000 replications) >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *E. coli* ATCC 11775^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid composition (%) for strain ISL-39^T and other *Microbulbifer* species

Strains: 1, ISL-39^T; 2, *M. hydrolyticus* DSM 11525^T; 3, *M. salipaludis* SM-1^T; 4, *M. elongatus* DSM 6810^T; 5, *M. maritimus* TF-17^T. Data for 2–5 are from Yoon *et al.* (2004). Fatty acids representing less than 0.5% in all strains have been omitted. The results presented were obtained using identical methods. –, Not detected.

Fatty acid	1	2	3	4	5
Straight-chain fatty acids					
C _{10:0}	1.6	1.7	2.4	1.6	1.3
C _{14:0}	0.9	1.2	2.6	0.7	1.0
C _{15:0}	1.5	1.5	1.7	0.9	1.6
C _{16:0}	12.6	11.4	16.3	7.1	8.7
C _{17:0}	2.6	2.9	2.2	2.5	1.3
C _{18:0}	0.8	1.6	1.4	1.2	–
Branched fatty acids					
iso-C _{11:0}	6.7	5.7	4.8	6.5	10.0
iso-C _{15:0}	21.7	24.4	19.4	20.7	25.9
iso-C _{15:1} F*	0.4	1.0	0.7	1.0	0.8
iso-C _{16:0}	0.3	–	–	0.5	–
iso-C _{17:0}	10.0	10.4	5.5	9.9	6.9
anteiso-C _{17:0}	0.3	–	–	0.8	–
iso-C _{17:1} ω9c	8.6	10.1	9.5	11.3	12.6
Unsaturated fatty acids					
C _{17:1} ω8c	0.5	0.5	1.0	1.8	–
C _{18:1} ω5c	–	–	0.7	–	–
C _{18:1} ω7c	6.5	8.9	11.8	16.3	5.6
Hydroxy fatty acids					
C _{10:0} 3-OH	1.4	1.0	1.2	1.6	1.7
C _{16:0} 2-OH	–	–	0.9	–	–
iso-C _{11:0} 3-OH	8.9	6.2	5.7	7.7	14.2
iso-C _{17:0} 3-OH	–	–	0.9	–	–
Cyclo fatty acids					
C _{17:0} cyclo	6.3	5.7	–	–	2.3
C _{19:0} cyclo ω8c	5.3	1.0	–	–	1.4
Summed features†					
3	2.4	2.7	7.1	6.0	2.2
4	–	–	1.7	–	–

*The double-bond position, indicated by a capital letter, is unknown.

†Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained one or more of the following fatty acids: iso-C_{15:0} 2-OH and/or C_{16:1} ω7c. Summed feature 4 contained one or more of the following fatty acids: anteiso-C_{17:1} B and/or iso-C_{17:1} I.

iso-C_{17:0}. The DNA G+C content is 57.7 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, ISL-39^T (=KCTC 12973^T=CCUG 54356^T), was isolated from a marine solar saltern of the Yellow Sea in Korea.

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